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| Author(s) | Hisa, Yusuke; Suzuki, Haruka; Atsumi, Go; Choi, Sun Hee; Nakahara, Kenji S.; Uyeda, Ichiro |
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**P3N-PIPO of *Clover yellow vein virus* exacerbates symptoms in pea infected with *White clover mosaic virus* and is implicated in viral synergism**

Yusuke Hisa[a, 1], Haruka Suzuki[a, 1], Go Atsumi[b], Sun Hee Choi[a], Kenji S. Nakahara[a, *], and Ichiro Uyeda[a]

[a] Pathogen-Plant Interactions Group, Plant Breeding Science, Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

[b] Iwate Biotechnology Research Center, Kitakami 024-0003, Iwate, Japan

*Corresponding author. Tel: +81 11 706 2490; Fax: +81 11 706 2483

*E-mail address*: knakahar@res.agr.hokudai.ac.jp

[1] These two authors contributed equally to this paper.

**Abstract**

Mixed infection of pea (*Pisum sativum*) with *Clover yellow vein virus* (ClYVV) and *White clover mosaic virus* (WClMV) led to more severe disease symptoms (a phenomenon called viral synergism). Similar to the mixed ClYVV/WClMV infection, a WClMV-based vector encoding P3N-PIPO of ClYVV exacerbated the disease symptoms. Infection with the WClMV vector encoding ClYVV HC-Pro (a suppressor of RNA silencing involved in potyviral synergisms), also resulted in more severe symptoms, although to a lesser extent than infection with the vector encoding P3N-PIPO. Viral genomic RNA accumulated soon after inoculation (at 2 and 4 days) at higher levels in leaves inoculated with WClMV encoding HC-Pro but at lower levels in leaves inoculated with WClMV encoding P3N-PIPO than in peas infected with WClMV encoding GFP. Our results suggest that ClYVV P3N-PIPO is involved in the synergism between ClYVV and WClMV during pea infection through an unknown mechanism different from suppression of RNA silencing.

Keywords: Synergism, *Potyvirus*, *Potexvirus*, P3N-PIPO, HC-Pro, Plant virus, Virulence

## Introduction

Multiple infections by plant viruses are common (DaPalma et al., 2010; Matthews, 1991). Complex facilitative and antagonistic interactions of the viruses in mixed infections result in unpredictable disease phenotypes in infected plants (Hammond et al., 1999; Syller, 2012). Antagonistic interactions between closely related viruses lead to cross-protection and mutual exclusion. Facilitative interactions between unrelated viruses lead to synergism, whereby one virus enhances the virulence or complements the defects of the other one, and helps it to replicate, to move systemically or to be transmitted by vector organisms; the resulting effect in mixed infections becomes greater than the sum of the individual effects of the two viruses. Viral synergism may increase crop damage and yield loss. Naturally occurring mixed viral infections cause severe diseases, such as maize lethal necrosis (Scheets, 1998), cassava mosaic disease (Pita et al., 2001), sweet potato virus disease (Mukasa et al., 2006; Untiveros et al., 2007), garlic mosaic disease (Lot et al., 1998), and a chili pepper disease known as "rizado amarillo" (Rentería-Canett et al., 2011).

The best characterized synergistic interaction of plant viruses is that between the members of the *Potyviridae* family *Potato virus Y* (PVY, genus *Potyvirus*) and *Potato virus X* (PVX, genus *Potexvirus*). Mixed infections by these viruses enhance the disease symptoms in tobacco, and result in an increased accumulation of PVX in comparison with single PVX infection (Goodman and Ross, 1974; Rochow and Ross, 1955), although the level of PVY does not increase (Vance, 1991). PVX can synergistically interact with other potyviruses, such as *Pepper mottle virus*, *Tobacco vein mottling virus* (TVMV), and *Tobacco etch virus* (TEV) (Vance et al., 1995). A number of potyviruses synergistically enhance multiplication of other unrelated viruses in the following combinations: PVY with *Potato leafroll virus* (Barker, 1987); *Soybean mosaic virus* with *Bean pod mottle virus* or *Cowpea mosaic virus*

(Anjos et al., 1992; Calvert and Ghabrial, 1983); *Zucchini yellow mosaic virus* with *Cucumber mosaic virus* (CMV) or *Cucurbit aphid-borne yellows virus* (Bourdin and Lecoq, 1994; Poolpol and Inouye, 1986); *Turnip mosaic virus* (TuMV) with CMV (Sano and Kojima, 1989); *Watermelon mosaic virus* with CMV (Wang et al., 2002); and *Blackeye cowpea mosaic virus* with CMV (Anderson et al., 1996).

The helper component protease (HC-Pro), a multifunctional protein with *cis*-proteolytic activity, is involved in viral movement and in transmission by aphids, and mediates viral synergism. PVX behaved synergistically in transgenic tobacco plants expressing the 5′-proximal region of TVMV or TEV encoding protease-1 (P1), HC-Pro, and protein-3 (P3) (but not other regions) (Vance et al., 1995). A PVX vector encoding HC-Pro enhanced disease severity and increased the level of PVX sense genomic RNA in tobacco, whereas a combination of P1 and HC-Pro additionally increased the level of PVX antisense genomic RNA, suggesting an accessory contribution of P1 to synergism (Pruss et al., 1997).

Suppression of RNA silencing in plants by HC-Pro is considered to be involved in viral synergism, as evidenced by the following studies. Not only HC-Pro but also other viral RNA silencing suppressors (RSSs) enhance PVX virulence when co-expressed (Moissiard and Voinnet, 2004; Scholthof et al., 1995; Voinnet et al., 1999), and are involved in viral synergism (Latham and Wilson, 2008; Ryang et al., 2004; Vanitharani et al., 2004). The disease synergism between *Wheat streak mosaic virus* (genus *Tritimovirus*) and *Maize chlorotic mottle virus* (genus *Machlomovirus*) does not require HC-Pro of *Wheat streak mosaic virus* (Stenger et al., 2007). In tritimoviruses, P1 but not HC-Pro has RSS activity, which implies the involvement of P1 in synergism (Young et al., 2012). However, various phenotypes of synergistic interaction between diverse virus combinations have been described (Syller, 2012; Takeshita et al., 2012). Mechanisms other than RNA silencing suppression may also mediate viral synergism (Caracuel et al., 2012; Latham and Wilson, 2008; Omarov et al., 2005).

*White clover mosaic virus* (WClMV, genus *Potexvirus*) causes chlorotic mottling and mosaic in clover species and other legumes worldwide (ICTVdB Management, 2006). Its genome (positive-sense, single-stranded RNA; Koenig, 1971), encoding a replicase, three movement proteins, and a coat protein (CP), has been cloned and used to construct an infectious clone and to develop a viral expression vector (Beck et al., 1990; Forster et al., 1988). We have also recently developed a WClMV vector derived from WClMV-RC isolated from red clover (Ido et al., 2012).

*Clover yellow vein virus* (ClYVV; genus *Potyvirus*) can infect both monocot and dicot plants. It causes chlorotic and necrotic symptoms in legumes, including white clover (*Trifolium repens*), broad bean (*Vicia faba*), and pea (*Pisum sativum*) (Bos et al., 1974; Hollings and Nariani, 1965; Tracy et al., 1992; Uyeda, 1992). ClYVV has a single-stranded RNA attached to the genome-linked protein at the 5′- end and a poly(A) tail. Its genome has one large open reading frame (ORF), which is translated as a polyprotein, which is further processed by *cis*- and *trans*-proteolytic activities into a number of functional mature proteins, including P1, HC-Pro, P3, 6K1, cylindrical inclusion body, 6K2, the C-terminal part of the nuclear inclusion protein *a*, viral genome-liked protein, nuclear inclusion protein b, and CP (see Fig. 1A). ClYVV HC-Pro suppresses RNA silencing (Yambao et al., 2008). A small ORF, called *Pretty Interesting Potyviridae* ORF (*PIPO*), was recently identified in the P3 cistron at +2 (or −1) frame relative to the large ORF (Chung et al., 2008). PIPO does not have its own translation initiation codon, but has the $G_{1\text{-}2}A_{6\text{-}7}$ sequence, which appears to cause a ribosomal frameshift (FS) of the large ORF during translation or transcriptional slippage (TS) during replication of the viral genome. Thus, two proteins are produced from the P3 cistron in

ClYVV-infected plants: P3 and a fusion protein with the N-terminal part of P3 (P3N-PIPO).

Recently, we found that P3 cistron products act as virulence determinants in pea carrying the recessive resistance gene (*cyv1*) against ClYVV (Choi et al., 2013). In the study, we used the WClMV vectors to produce either exclusively or predominantly P3 or P3N-PIPO to dissect the involvement of P3 cistron products in the *cyv1* resistance. We found that WClMV producing P3N-PIPO resulted in the most severe symptoms in inoculated susceptible peas among several constructs tested, including WClMV producing HC-Pro. This implies that P3N-PIPO (along with HC-Pro) is involved in synergism between ClYVV and WClMV if the synergism between these viruses occurs in pea.

## Results and discussion

### *Effect of ClYVV P3 cistron products on WClMV virulence in pea*

To construct WClMV vectors for exclusive production of P3 or P3N-PIPO, we introduced a stop codon downstream of the $G_2A_6$ motif in the P3N-PIPO or P3 frame, respectively; in the latter case, we also inserted an additional G to create a $G_2AGA_5$ motif (Fig. 1) and integrated a truncated P3 cistron cDNA fragment (from the beginning of the cistron to the end of the PIPO ORF) into the WClMV vector to ensure that the P3 protein is not produced. Our recent study revealed that the genomic sequence of the P3 cistron was sufficient to produce the P3N-PIPO protein through FS or TS (Choi et al., 2013). We also constructed WClMV vectors containing the unmodified P3 cistron, or P3 with $G_2A_7$ instead of $G_2A_6$, which were expected to produce mainly P3 with trace amounts of P3N-PIPO ($P3^{+P3N\text{-}PIPO}$), or P3N-PIPO with trace amounts of P3 ($P3N\text{-}PIPO^{+P3}$) as long as -2 (or +1) FS or

TS occurs, respectively (Fig. 1).

We tried to detect the production of corresponding proteins in infected pea leaves by western blotting with specific antibodies (Choi et al., 2013), but failed to detect either, even in samples in which their exclusive production was expected. Therefore, we added the Flag tag to the N-terminus of the P3 cistron products (Fig. S1C). Using the anti-Flag antibody, we were unable to detect Flag-P3N-PIPO; Flag-P3 could not be definitely identified, because a similar band of lower intensity was also observed in mock-inoculated and Flag-P3N-PIPO-producing plants (Fig. S1A). Nevertheless, Flag-P3N-PIPO was clearly detected with the anti-Flag antibody when the cDNA encoding P3N-PIPO was replaced in a binary vector and its encoding P3N-PIPO was transiently expressed by agroinfiltration (Fig. S1B). The green fluorescent protein (GFP) and other Flag-tagged proteins could be produced by using the WClMV vector used in this study (Atsumi et al., 2012; Ido et al., 2012). We confirmed the retention and presumable expression of all WClMV constructs by RT-PCR in the following experiments.

We inoculated these WClMV constructs and a construct producing GFP, which was constructed previously (Ido et al., 2012), into pea plants susceptible to ClYVV and WClMV. Approximately a week after inoculation of the third leaves (from the bottom), chlorosis and vein clearing were usually observed in fifth leaves of plants infected with WClMV expressing GFP (Fig. 2A), similar to our earlier observations (Ido et al., 2012). Inoculation with the GFP construct and with all P3 constructs (except P3N-PIPO) resulted in comparable stunting and other symptoms in infected plants (Fig. 2A, C), whereas inoculation with the WClMV vector producing P3N-PIPO resulted in more severe stunting leaf yellowing and eventual blasting. The P3N-PIPO$^{+P3}$ construct resulted in more severe symptoms than P3$^{+P3N\text{-}PIPO}$. We confirmed by RT-PCR and northern blotting the WClMV infection and retention of the P3 cistrons in all progeny viruses (Fig. 2B, D). RT-PCR amplified a shorter DNA fragment from the vector designed to produce P3N-PIPO only versus from the other vectors because the P3 cDNA used in this construct was truncated. These results indicate that heterologous expression of ClYVV P3N-PIPO enhanced WClMV virulence.

*Synergistic disease enhancement by mixed infection with ClYVV and WClMV*

We either inoculated each of ClYVV and WClMV into the opposite blades of the same leaf pair, or infected the plants with either of the viruses (Fig. 2E). As expected, plants infected with both viruses had more severe stunting, yellowing, and blasting of uninoculated upper leaves than plants infected with a single virus. The mixed infection did not alter the accumulation of the WClMV genome (Fig. 2F). The absence of the effect on genome accumulation is perhaps not surprising: the synergism between PVX and TEV or PVY is accompanied by an increased PVX accumulation in doubly infected *N. tabacum* but not in *N. benthamiana* (Gonzalez-Jara et al., 2004); thus, viral genome accumulation in mixed infections may depend on the host plant species.

*Exacerbation of disease symptoms in peas infected with WClMV expressing ClYVV HC-Pro depends on its RNA silencing suppression activity*

To examine whether HC-Pro of ClYVV is involved in synergism between ClYVV and WClMV, we inoculated the WClMV vectors encoding GFP, wild-type HC-Pro, or HC-Pro$^{D193Y}$, a mutant with greatly reduced RSS function (Yambao et al., 2008). Plants infected with the HC-Pro construct had more severe stunting as well as deformation and mosaic of leaves than plants infected with the GFP construct (Fig. 3A). However, symptom exacerbation almost disappeared in plants infected with the HC-Pro$^{D193Y}$ construct. Similar exacerbation of symptoms was observed in plants infected with WClMV encoding P19, a strong tombusvirus RSS (Fig. 3C, D), which was constructed in our previous study (Atsumi et al., 2012). Thus, the effect of HC-Pro could be attributed to its RSS function. This conclusion is supported by our previous observation that RSSs from other viruses complemented the attenuation of ClYVV virulence in pea due to the HC-Pro$^{D193Y}$ point mutation (Atsumi et al., 2012). Although both HC-Pro and P19 enhanced the WClMV virulence, yellowing and eventual leaf blasting were not observed.

*Quantitative comparison of virulence of WClMVs expressing P3N-PIPO, HC-Pro and GFP in infected pea plants*

Using northern blotting analysis, we tested the accumulation of the WClMV genomic RNA in inoculated third upper leaves soon after inoculation, and found that significantly less viral genome accumulated 4 days post-inoculation (dpi) in inoculated leaves of pea plants infected with WClMV expressing P3N-PIPO than in those of plants infected with WClMV expressing GFP (Fig. 4A–C). In contrast, more viral genome accumulated upon inoculation with WClMV expressing HC-Pro than the GFP control (Fig. 4A–C). This suggests that P3N-PIPO did not enhance WClMV virulence by increasing WClMV accumulation as HC-Pro did. Plant stunting by WClMV with both P3N-PIPO and HC-Pro was statistically significant (Fig. 4D, E).

*Possible mechanisms of synergism involving P3N-PIPO*

Our results suggest that the synergistic exacerbation of symptoms by mixed infection with ClYVV and WClMV involved P3N-PIPO in addition to HC-Pro. We also confirmed that tombusviral RSSs, P19 and HC-Pro (but not HC-Pro$^{D193Y}$), enhanced WClMV virulence. Thus, one likely possibility is that P3N-PIPO possesses RSS activity. RNA silencing is a general antiviral defense against diverse viruses, including plant RNA and DNA viruses. Thus, in mixed infections, inhibition of antiviral RNA silencing by one virus is beneficial for another virus, resulting in increased accumulation or enhanced virulence of the latter. Whether P3N-PIPO from ClYVV has RSS activity remains to be established. No RSS activity of P3N-PIPO from a tritimovirus could be detected (Young et al., 2012).

Alternatively, P3N-PIPO might enhance WClMV replication or spread. Movement of potexviruses is facilitated or transcomplemented by movement proteins from *Dianthovirus*, *Umbravirus*, *Bromovirus*, *Cucumovirus*, and *Tobamovirus* (Latham and Wilson, 2008). The identity of the movement protein in potyviruses remained ambiguous until the recent discovery of P3N-PIPO. Mutant potyviruses (TuMV and *Soybean mosaic virus*) deficient in the production of P3N-PIPO lose their ability to move and their pathogenicity (Chung et al., 2008; Wen and Hajimorad, 2010). P3N-PIPO is located at the plasmodesmata and recruits the cylindrical inclusion protein by binding to it (Wei et al., 2010). Wei et al. (2010) proposed a model of cell-to-cell movement of potyviruses, which involves P3N-PIPO, the cylindrical inclusion protein, CP, and viral genomic RNA. A host membrane protein, PCaP1, interacts with P3N-PIPO, and TuMV spread and replication are attenuated when PCaP1 is silenced (Vijayapalani et al., 2012). Transiently expressed P3N-PIPO fused with GFP spreads to adjacent cells without viral infection (Vijayapalani et al., 2012). These studies imply the possibility that P3N-PIPO enhances the WClMV virulence by facilitating its spread in pea plants.

Neither mixed infection with ClYVV and WClMV nor single infection with WClMV producing P3N-PIPO increased the accumulation of WClMV. Symptoms such as yellowing and blasting of upper leaves, which were observed in peas doubly infected with ClYVV and WClMV or infected with WClMV producing P3N-PIPO, were not observed in plants infected with WClMV encoding RSSs (HC-Pro and P19). These results raise a possibility that P3N-PIPO contributes to synergism through an unknown mechanism that enhances WClMV virulence and that is different from suppression of RNA silencing as in the case of HC-Pro. Further studies are required to understand how P3N-PIPO contributes to the synergism between ClYVV and WClMV. P3N-PIPO might activate host defense responses, leading to the reduced accumulation of WClMV genome in leaves (Fig. 4A–C). Although single infection with ClYVV did not induce systemic necrosis in the pea line used in this study (PI 250438), it does so in many pea and broad bean lines. We have recently shown that activation of salicylic acid–mediated defense responses is required for systemic necrosis in pea infected with ClYVV (Atsumi et al., 2009); the viral determinants of this necrosis have been mapped to the region including HC-Pro (Yambao et al., 2008) and P3 (Atsumi et al., unpublished). Therefore, P3N-PIPO is a candidate for the protein involved in induction of systemic necrosis by ClYVV infection, and might activate host defense responses. Although this does not result in necrosis in PI 250438, it may still result in symptom exacerbation in PI 250438 plants infected with WClMV expressing P3N-PIPO.

## Conclusion

We revalidated the synergistic interaction between ClYVV (*Potyvirus*) and

WClMV (*Potexvirus*) by analyzing the mixed infection of pea with these viruses and the infection with WClMV producing ClYVV HC-Pro. WClMV producing ClYVV P3N-PIPO induced similar disease symptoms as the double infection, suggesting that P3N-PIPO from ClYVV enhances WClMV virulence in infected peas and thus may be involved in synergism between these viruses. Because P3N-PIPO reduced the accumulation of WClMV genomic RNA in inoculated leaves, whereas HC-Pro increased it, the enhanced WClMV virulence by P3N-PIPO perhaps involves a mechanism different from suppression of RNA silencing too. This mechanism might be activation of host defenses in response to P3N-PIPO.

## Materials and methods

### *Construction of the WClMV vectors to produce the ClYVV proteins*

The P3 cistron of ClYVV was used either unmodified to produce predominantly P3 ($P3^{+P3N\text{-}PIPO}$) or modified to produce (1) exclusively P3, (2) exclusively P3N-PIPO, or (3) predominantly P3N-PIPO ($P3N\text{-}PIPO^{+P3}$) (see Fig. 1B). cDNA was reverse-transcribed from RNA of the ClYVV RB (Choi et al., 2013) (isolated from infected peas) as a template. $P3^{+P3N\text{-}PIPO}$ was obtained by PCR with primers P3s1 (5′-aaaactagtatgggcaaatcattgacaggg-3′) and P3as2 (5′-aaaactcgagctattccatgacaaaccact-3′) and a DNA polymerase, KOD-plus2 neo (Toyobo, Osaka, Japan). Modifications were introduced by a two-step PCR as follows. For exclusive production of P3, a stop codon was introduced just after the conserved $G_2A_6$ motif in the PIPO frame. The fragment from the 5′-terminus to the beginning of the PIPO ORF was amplified with primers P3s1 and P3as1 (5′-ctaaatcctcagcccaaattttttcc-3′); the fragment from the beginning of the PIPO ORF to the 3′-terminus of the P3 cistron was amplified with primers P3s2 (5′-ggaaaaaatttgggctgaggatttag-3′) and P3as2. The PCR products were purified by agarose gel electrophoresis and mixed, and the modified P3 cistron was amplified from the mixture by the second PCR with primers P3s1 and P3as2. For the exclusive production of P3N-PIPO, the conserved motif was modified into GGAGAAAAA, and a stop codon was introduced just after the conserved motif in the P3 frame. The following primers were used for a two-step PCR: P3s1, P3as3 (5′-aaatcctctgcctaaatttttctcc-3′), P3s3 (5′-ggagaaaaatttaggcagaggattt-3′) and P3as5 (5′-aaaactcgagcacttcttacttacttgttgcgac-3′). For P3N-PIPO$^{+P3}$ production, the conserved motif was modified into GGAAAAAAA by a two-step PCR with the following primers: P3s1, P3as4 (5′-taaatcctctgcccaaattttttttcc-3′), P3s4 (5′-ggaaaaaaaatttgggcagaggattta-3′) and P3as2. The modified and unmodified P3 fragments were digested with *Spe*I and *Xho*I were inserted into the WClMV vector (Ido et al., 2012). We also constructed the WClMV vectors encoding the Flag-tagged P3 cistron products as described above with the primer Flag-P3s1 (5′-aaaactagtatggactacaaggacgacgatgcaagatgggcaaatca-3′) instead of P3s1. The HC-Pro and the point mutant HC-Pro$^{D193Y}$ cDNA fragments were amplified from the ClYVV infectious clones (Yambao et al., 2008) by PCR with the primers: 5′-aaagctagcatgtctgcaggagatttg-3′ and 5′-aaaactagtctaaccaactctgtaaaacttcaaatctga-3′. The HC-Pro fragments were digested with *Nhe*I and *Spe*I and inserted into the WClMV vector (Ido et al., 2012). Their nucleotide sequences were confirmed by sequencing.

*Inoculation of pea plants and observation of the infection symptoms*

The constructs and ClYVV RB (Choi et al., 2013) were mechanically inoculated into susceptible pea plants (line PI 250438). Approximately a week later, the upper leaves with disease symptoms were collected (to be used as inoculum), and stored at −80°C. To test pea responses to the constructs, the third leaves from the bottom were mechanically inoculated with stored infected leaves. Plants were incubated for 2–3 weeks in a growth chamber (16 h light, 8 h dark, 23 °C). For each plant, photographs were taken of the fifth leaf from the bottom and of the whole plant body at 14 dpi.

*Detection of WClMV genomic RNA and transgenes by northern blotting and RT-PCR*

Total RNA was extracted from the sixth leaves from the bottom of inoculated pea plants at 12 dpi in Trizol reagent (Life Technologies, Tokyo, Japan) according to the manufacturer's manual. Total RNA (2 μg) was fractionated in agarose gel containing formaldehyde, and transferred to Hybond-N nylon membrane (GE Healthcare Life Sciences, Germany) as described previously (Yambao et al., 2008). The WClMV genome was detected with a digoxigenin (DIG)-labeled cRNA probe that was antisense to the CP gene of WClMV (Ido et al., 2012). Chemiluminescent signals were detected by LAS-4000 mini PR Lumino-image analyzer (Fujifilm, Tokyo, Japan). Retention of the transgenes in WClMV progeny was confirmed by RT-PCR as described previously (Ido et al., 2012) with f (5′-taataggcgtatatcttctagt-3′) and r (5′-aagcgagaggcaagacgtcat-3′) primers (Fig. 1A).

*Western blotting*

ClYVV P3, P3N-PIPO and CP were detected by western blotting essentially as described previously (Nakahara et al., 2012) using polyclonal rabbit antibodies raised against them (Andrade et al., 2007; Choi et al., 2013). Flag-tagged P3 and P3N-PIPO produced from the WClMV vectors in infected peas were detected with anti-Flag M2 monoclonal antibody (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany). Viral titer was determined by measuring luminance with Multi Gauge software (Fujifilm, Tokyo, Japan) in Fig. 4C. rRNA was quantified by using ImageJ software in Fig. 4C.

*Agrobacterium-mediated transient expression of P3N-PIPO*

The cDNA fragment encoding Flag-tagged P3N-PIPO, flanked by *Xho*I and *Spe*I sites, was prepared by PCR with the primers 5′-agctactagtTTACTTGTTGCGACCATTCTC-3′ and 5′-atgcctcgagATGGACTACAAGGATGACGATGACAAGggcaaatcattgacagggcag-3′ by using the WClMV vector encoding P3N-PIPO as a template. The fragment obtained was cloned into binary vector pTA7001 to yield pTA/FLAG-P3NPIPObr2, which was used to transform the LBA4404 strain of *A. tumefaciens*; the transformants were infiltrated into *N. benthamiana* leaves according to Yambao et al. (2008). P3N-PIPO expression was induced with dexamethasone (DEX) as described previously (Aoyama and Chua, 1997).

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## Figure legends

**Fig. 1.** Construction of the *White clover mosaic virus* (WClMV) vectors containing the P3 cistrons of the *Clover yellow vein virus* (ClYVV). (A) Genome structures of ClYVV and the WClMV vectors; (B) Unmodified and modified P3 cistrons inserted in the WClMV vectors. The PIPO ORF is embedded in the P3 cistron without a translation initiation codon. Thus, the P3 cistron encodes not only P3 but also P3N-PIPO, which is composed of the N-terminal part

of P3 followed by PIPO and arises through ribosomal frameshift (FS) or transcriptional slippage (TS) at the conserved motif $G_2A_6$. Primers f and r were used for RT-PCR to confirm the retention of the transgene in the progeny of the WClMV vectors (see Materials and Methods). The P3 cistron in the WClMV vectors (black box) was used either unmodified (to produce mainly P3 with a small amount of P3N-PIPO: $P3^{+\text{ P3N-PIPO}}$) or modified to produce either P3 or P3N-PIPO exclusively, or to produce mainly P3N-PIPO with a small amount of P3 as long as –2 (or +1) FS or TS occurs at $G_2A_7$ (P3N-$PIPO^{+P3}$).

**Fig. 2.** Responses of susceptible pea plants (line PI 250438) to single or mixed infection with ClYVV and WClMV. (A) Upper panel: whole plants infected with WClMV encoding GFP, unmodified P3 cistron ($P3^{+P3N\text{-}PIPO}$), or modified P3 cistron that produces P3 or P3N-PIPO exclusively, or buffer-inoculated (mock) at 14 days post inoculation (dpi). Lower panel: the uninoculated fifth leaves from bottom. Orange bars mark the tops of pea plants inoculated with each of the constructs. A white scale bar indicates 10 mm. (B) Upper panel: accumulation of WClMV genomic RNA in the sixth leaves from the bottom of the plants shown in A, detected by northern blotting at 12 dpi. Middle panel: ribosomal RNA as a loading control. Lower panel: retention of the transgenes in the progeny of the WClMV vectors in the sixth leaves, confirmed by RT-PCR with the primers f and r (see Fig. 1A). Arrowheads, the expected positions of RT-PCR products. RT-PCR amplified a shorter DNA fragment from the vector encoding P3N-PIPO than the other vectors because the truncated P3 cDNA was used in this construct to ensure that the P3 protein is not produced. (C, D) The experiment was repeated with WClMV containing the modified P3 that produced mainly P3N-PIPO with a small amount of P3 (P3N-$PIPO^{+P3}$). (E) Upper panel: whole plants (at 14 dpi) infected with ClYVV, WClMV, both, or neither (buffer-inoculated; mock). Lower panel:

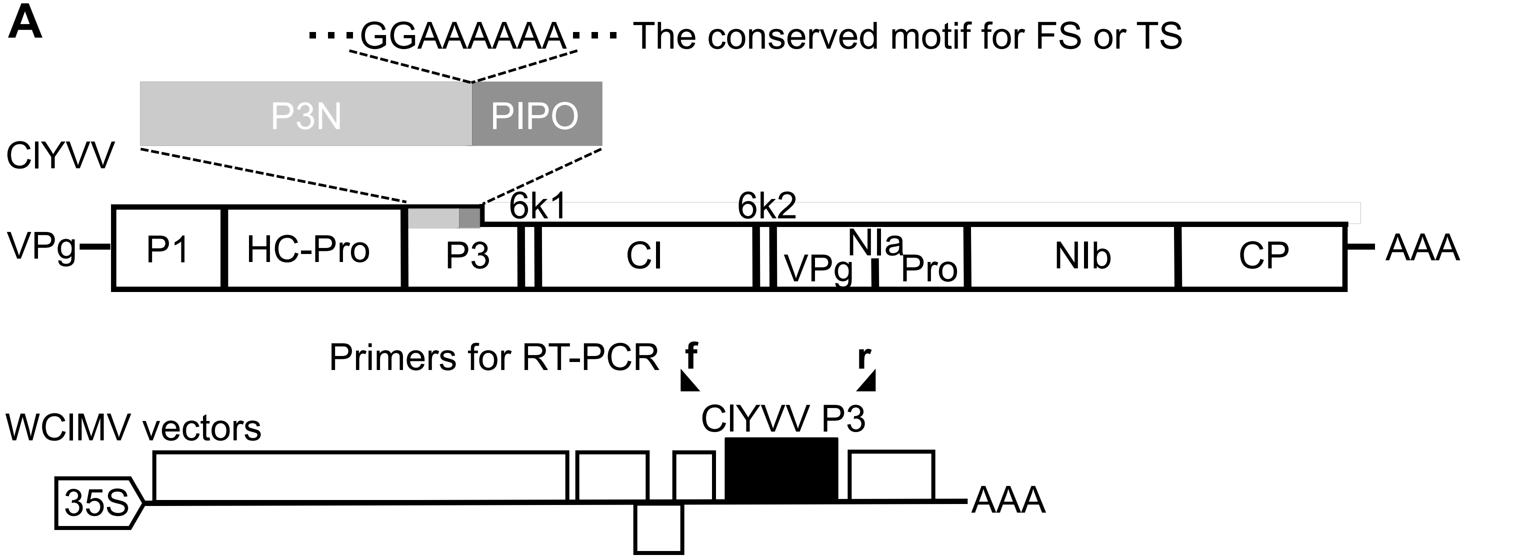
uninoculated fifth leaves from the bottom. (F) Upper panel: accumulation of WClMV genomic RNA (at 12 dpi) in the sixth leaves from the bottom of plants shown in A, detected by northern blotting. Upper middle panel: ribosomal RNA as a loading control. Lower middle panel: ClYVV infection confirmed by western blotting with anti-ClYVV CP antibody. Lower panel: Coomassie Brilliant Blue–stained gel as a loading control.

**Fig. 3.** Responses of susceptible pea plants to infection by WClMV vectors encoding viral RSSs or P3N-PIPO, or mixed infection by ClYVV and WClMV. (A) Upper panel: whole plants (at 14 dpi) infected with WClMV encoding GFP, ClYVV HC-Pro, the point mutant HC-Pro$^{D193Y}$, or buffer-inoculated (mock). Lower panel: uninoculated fifth leaves from the bottom. Orange bars mark the tops of pea plants inoculated with each of viruses. (B) Upper panel: accumulation of WClMV genomic RNA (at 12 dpi) in the sixth leaves from the bottom of plants shown in A, detected by northern blotting. Middle panel: ribosomal RNA as a loading control. Lower panel: retention of transgenes in the progeny of the WClMV vectors in the sixth leaves, confirmed by RT-PCR with the primers f and r. Arrowheads, the expected positions of RT-PCR products. (C) A similar experiment that included a mixed infection by ClYVV and WClMV, or by WClMV vectors carrying P3N-PIPO, HC-Pro, or tombusviral P19; retention of the P19 transgene in the progeny has been previously demonstrated (Atsumi et al., 2012). (D) Accumulation of WClMV genomic RNA was detected as in B.

**Fig. 4.** Quantitative analysis of susceptible pea plants inoculated with the WClMV vectors encoding ClYVV P3N-PIPO and ClYVV HC-Pro. (A) Relative WClMV titer and viral subgenome (sg) titer per ribosomal RNA (rRNA). (B) Relative WClMV titer per milligram of leaf weight of susceptible pea plants inoculated with WClMV encoding GFP, ClYVV

P3N-PIPO, ClYVV HC-Pro, or buffer (mock). Error bars, SD. *$P < 0.05$. (C) Accumulation of WClMV genomic RNA in the inoculated leaves detected by northern blotting at 2 dpi (upper part) and 4 dpi (lower part). Lower panels in each part show rRNA loading controls. Total RNA was extracted from inoculated leaves. Viral titer was determined by measuring luminance with Multi Gauge software. rRNA was quantified by using ImageJ software. At least four plants were used per analysis. (D) Heights and (E) weights of whole pea plants infected with the same constructs or buffer (mock) at 9 dpi. Error bars, SD. *$P < 0.05$.

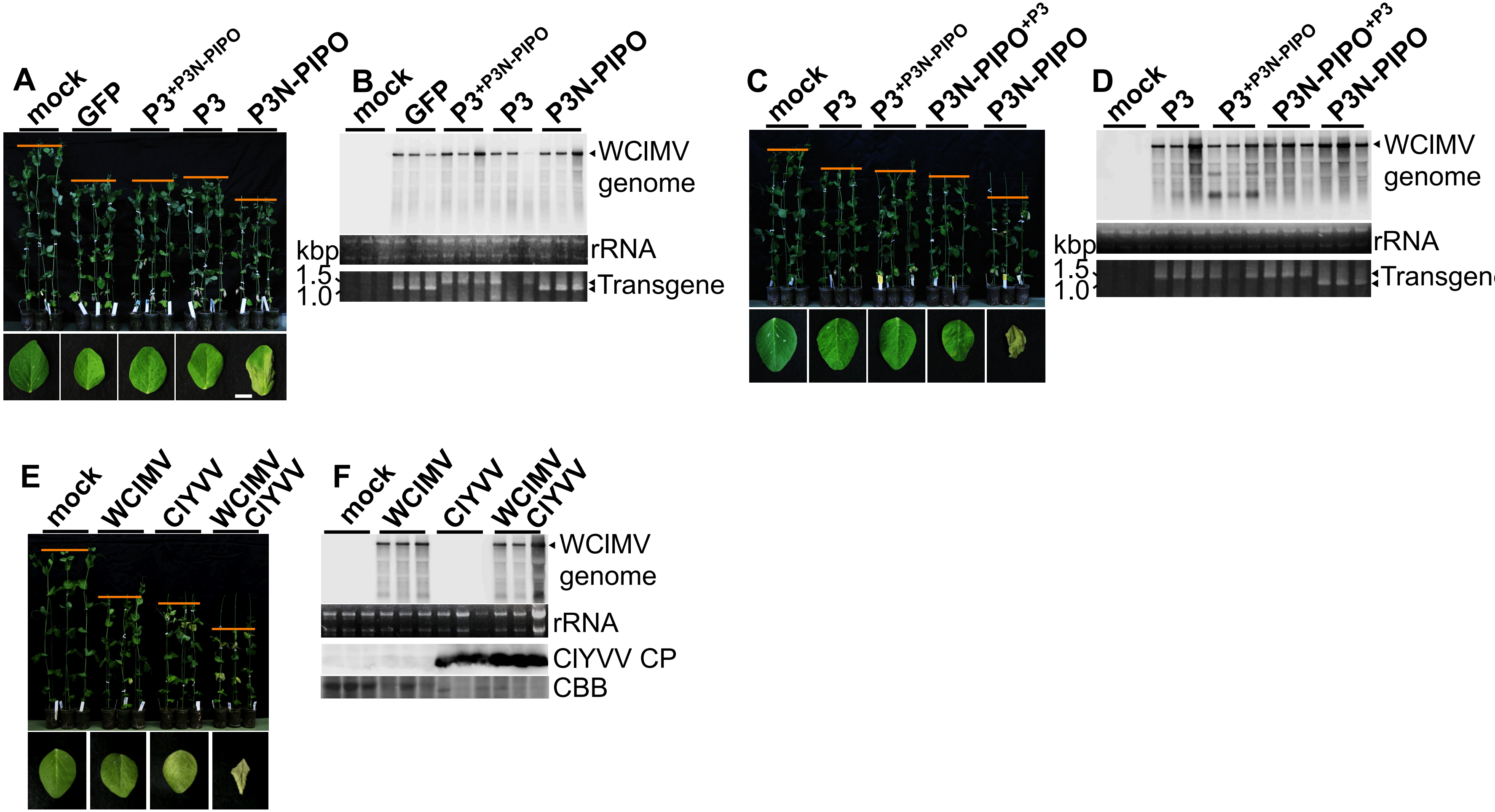
**A**



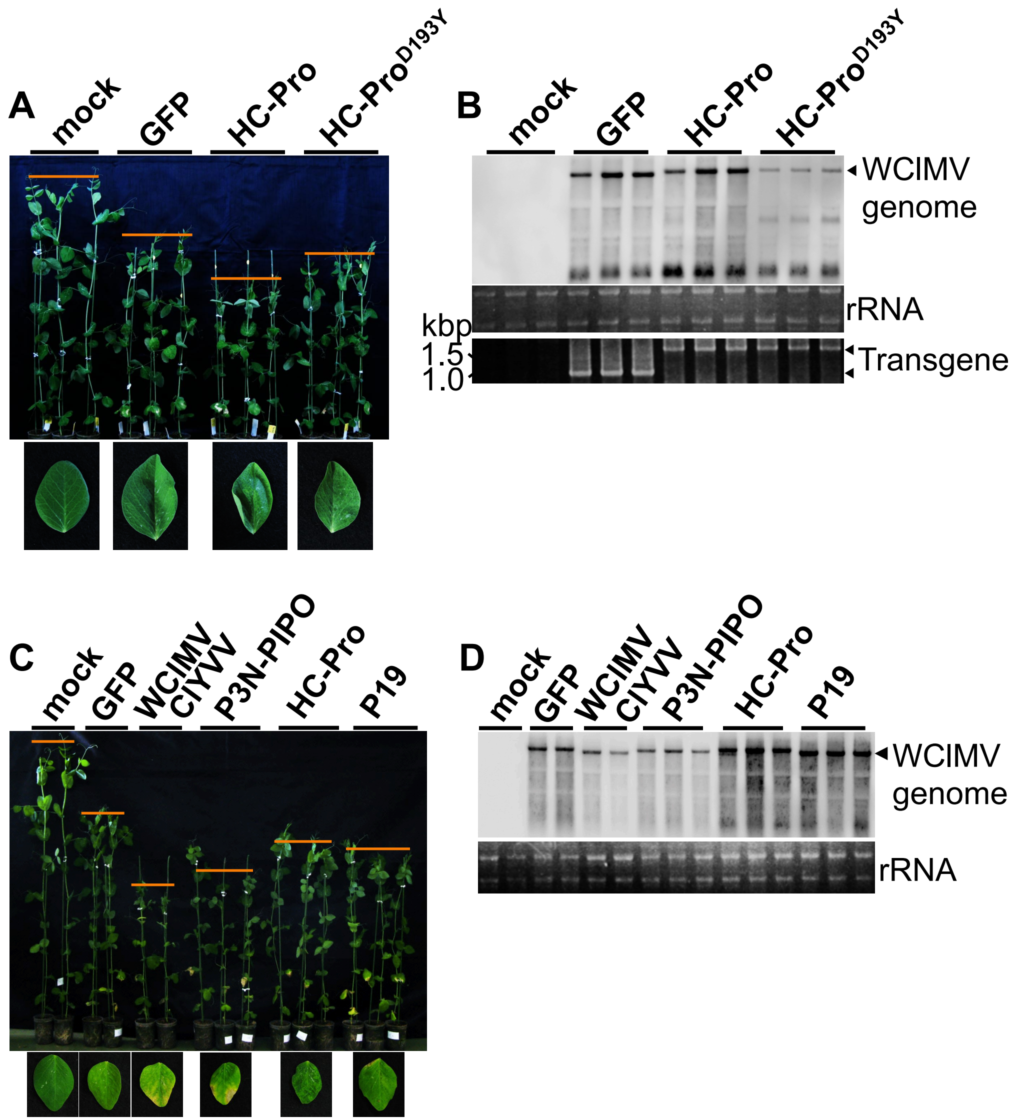

**B** WClMV vectors producing the proteins encoded in the P3 cistron

| Expected products | Modification of the conserved motif involved in FS or TS of P3 |
|---|---|
| | E K I W A E (the P3 amino acid sequence) |
| | K N L G R (the PIPO amino acid sequence) |
| P3+P3N-PIPO | GGA AAAAATTTGGGCAGAG (native nucleotide sequence) |
| P3N-PIPO | GGA**+G**AAAAATT<u>T**A**G</u>GCAGAG (<u>a stop codon</u> in the P3 frame) |
| P3 | GGA AAAAATTTGGGC<u>**T**GA</u>G (<u>a stop codon</u> in the PIPO frame) |
| P3N-PIPO+P3 | GGA**+A**AAAAATTTGGGCAGAG |

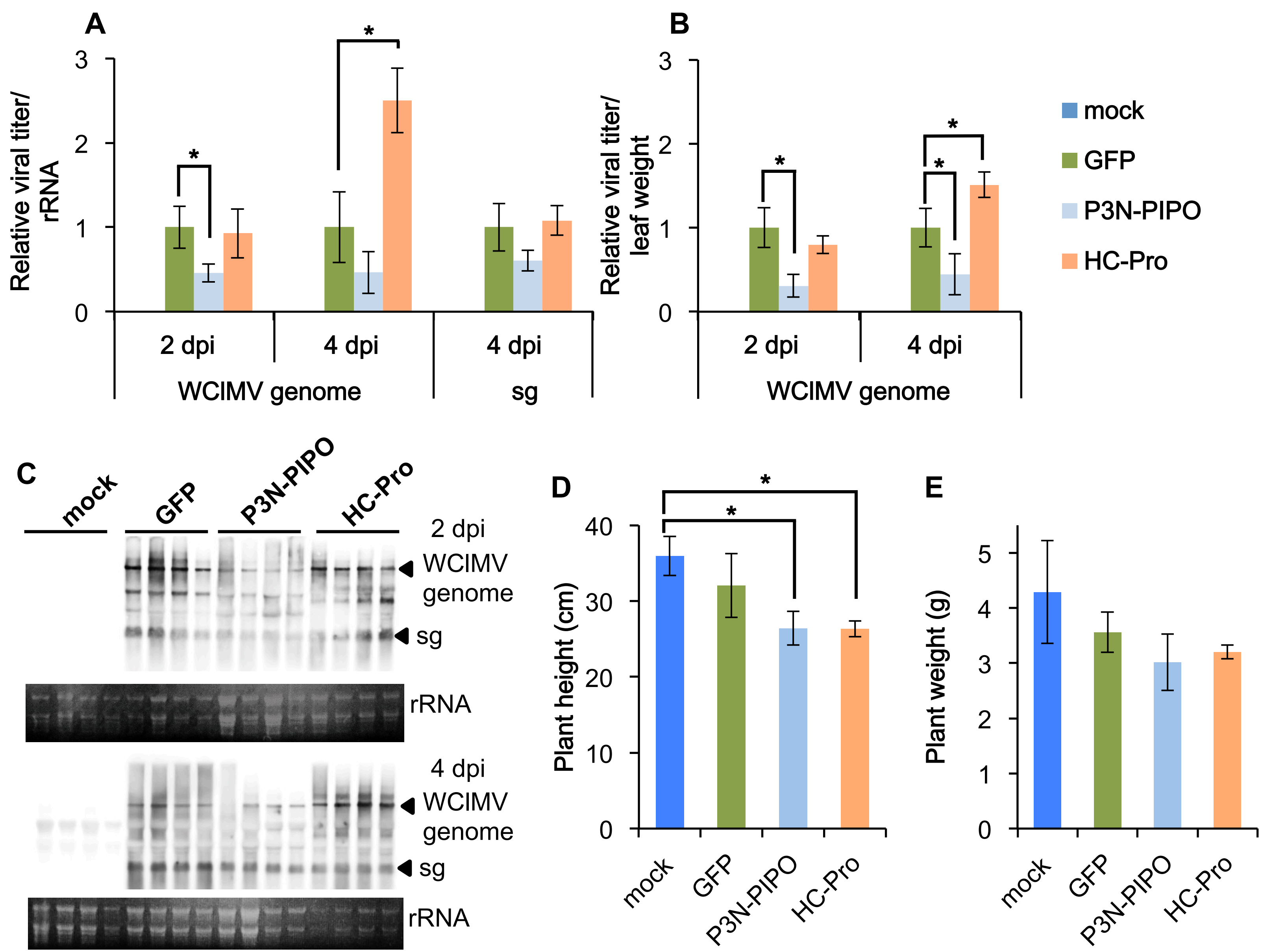
Hisa et al., Fig. 2



Hisa et al., Fig. 3



Hisa et al., Fig. 4



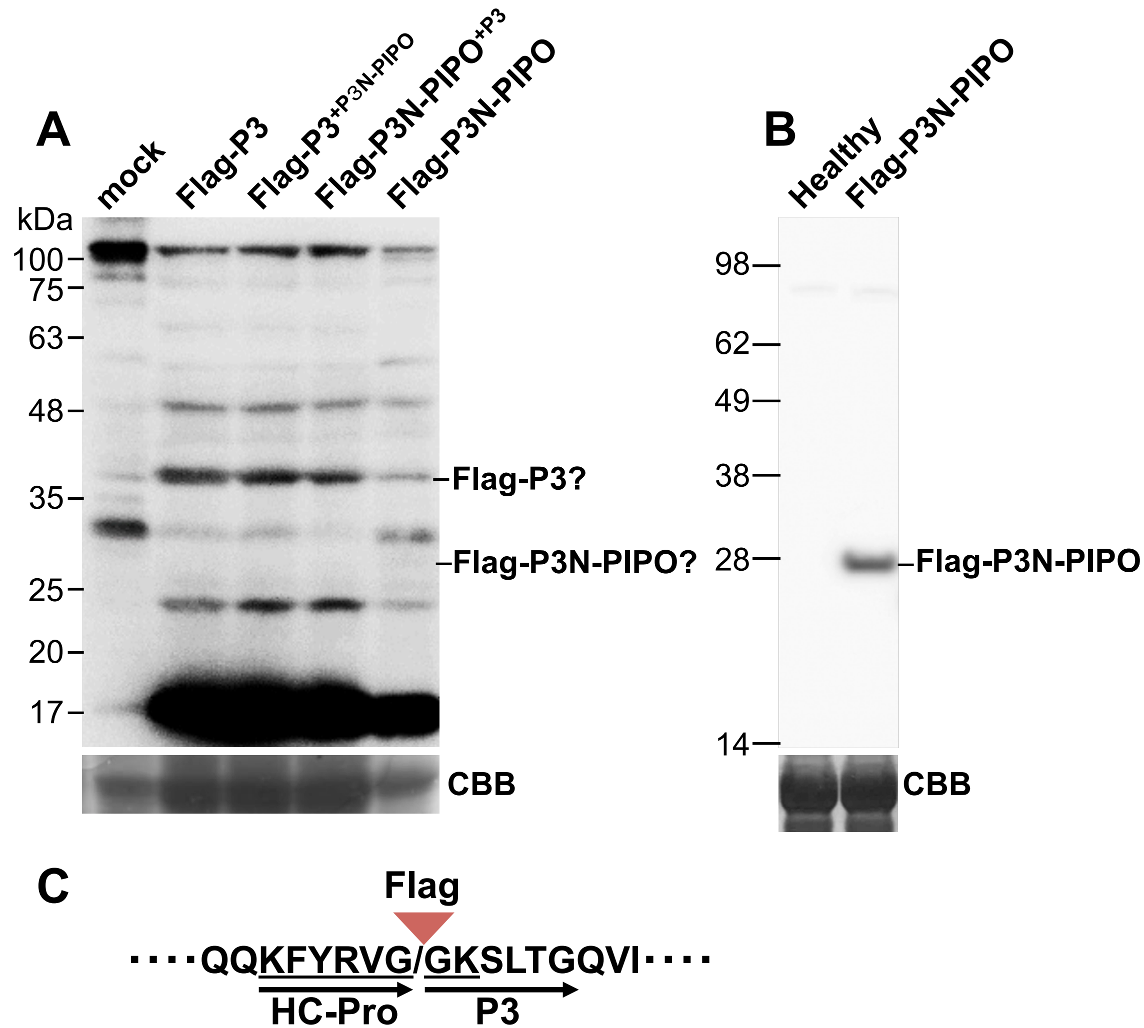



**Fig. S1**. Transient expression of Flag-tagged P3 constructs in (A) pea plants inoculated with WClMV and (B) agroinfiltrated N. benthamiana. (A) The WClMV vectors encoding the Flag-tagged P3 cistron (see Materials and Methods) were inoculated into pea plants. Total protein from the upper leaves showing symptoms was probed with an anti-Flag antibody on western blots. A Coomassie Brilliant Blue–stained gel (CBB) is shown as a loading control. (B) N. benthamiana leaves were transiently transformed by agrobacterium carrying a binary expression vector encoding P3N-PIPO. Expression of P3N-PIPO was induced with DEX as described previously (Aoyama and Chua, 1997). (C) The amino acid sequence of ClYVV around the putative cleavage site between HC-Pro and P3. The Flag tag was integrated at the beginning of the P3 amino acid sequence, as indicated by an arrowhead.